PRODUCTION OF ECTOMYCORRHIZAL FUNGUS INOCULUM

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INTRODUCTION

The need of many species of forest trees for ectomycorrhizae was initially observed when attempts to establish plantations of exotic pines in parts of the world deficient in the fungal partners routinely failed until the essential fungi were introduced. The need of pine and oak seedlings for ectomycorrhizae has also been demonstrated in the afforestation of former treeless areas, such as the grasslands of Russia and the Great Plains of the United States (51). Even on clearcut pine lands (64) or on amended adverse sites (62) nonmycorrhizal pine seedlings do not survive or grow well until indigenous fungi form ectomycorrhizae on their roots.

Ectomycorrhizae are formed by fungi belonging to the higher Basidiomycetes, Ascomycetes, and zygosporic Phycomycetes of the Endogonaceae (8,79,82). The host plants of these fungi are predominately trees belonging to the Pinaceae (pines, fir, larch, spruce, hemlock), Fagaceae (oak, chestnut, beech), Betulaceae (alder, birch), Salicaceae (poplar, willow), Juglandaceae (hickory, pecan), Myrtaceae (eucalyptus), Ericaceae (Arbutus), and other families (49).

Ectomycorrhizal fungi have been introduced into deficient soils in various inocula to provide seedlings with adequate ectomycorrhizae to create man-made forests. Most research on inoculation with ectomycorrhizal fungi has been based on two premises. First, any ectomycorrhizal association on roots of tree seedlings is far better than none. Success in correcting deficiencies has contributed greatly to our understanding of the importance of ectomycorrhizae to trees. Second, some species of ectomycorrhizal fungi on certain sites are more beneficial to trees than other fungal species that naturally occur on such sites. Much work in recent years with a few fungal species has been aimed at selecting, propagating, manipulating, and managing the more desirable fungal species to improve tree survival and growth.

The majority of past work on inoculation with ectomycorrhizal fungi has been done in nurseries for the production of bare-root or container-grown tree seedlings. Most future work with inoculations will undoubtedly continue to concentrate on seedling production in nurseries. However, the inoculation of tree seeds with spores of ectomycorrhizal fungi, in a manner similar to inoculation of legume seeds with Rhizobia, to improve seedling establishment following direct seeding operations could become a very important alternative to planting nursery grown seedlings, especially on the more remote sites or those of very rough terrains.

No ectomycorrhizal fungus has been shown to complete its life cycle in the absence of mycorrhizal association in a natural environment (10). Also, there is no evidence that these fungi grow saprophytically in a natural forest soil (69). Thus, in inoculation programs, since hyphae cannot grow from the inoculum to roots, inoculum must be placed in the rooting zone of seedlings where roots can grow into the inoculum. Once initial root infection takes place, extramatrical mycelium originating from the initial infection spreads rapidly to infect other roots.

Most ectomycorrhizal fungi produce above-ground sporophores. The spores produced in these sporophores are disseminated great distances by wind, rain, insects, and small animals. The more dense the tree stands supporting ectomycorrhizal fungi and the closer they are to nursery areas, the greater the chances are for rapid natural ectomycorrhizal development on the nursery seedlings. In the southern United States, ectomycorrhizae appear on nursery-grown tree seedlings in the spring as early as 6 to 8 weeks after seed germination. In nurseries surrounded by pine and oak stands that produce abundant sporophores of ectomycorrhizal fungi, early ectomycorrhizal development occurs even in nursery soils fumigated just a few days prior to seeding. The most common ectomycorrhizal fungus naturally colonizing fumigated nursery soil in the United States is Thelephora terrestris, a symbiont that produces numerous sporophores starting in early spring (45).

The majority of reports on inoculation techniques with ectomycorrhizal fungi involve Basidiomycetes on pines, oaks, and eucalypts. Several types of natural and laboratory-produced inocula and several methods of application have been used through the years. Many of the techniques have proven successful, others have not. Frequently, conflicting results are encountered (33).

Soil Inoculum

The most widely used natural inoculum, especially in developing countries, is soil or humus collected from established pine plantations (51). This soil inoculum is either mixed into the rooting medium (usually at 5 to 10 percent volume), broadcast 0.5 to 1 cm deep onto soil and watered into the soil around young seedlings, or suspended in water (1 kg soil in 20 ℓ of water) and poured onto seedlings. The latter two procedures are usually done three to four times during the nursery season. Better results are obtained with freshly collected soil than with soil collected and stockpiled for several months. In Morocco, soil inoculum collected from oak and eucalyptus stands is mixed with clay and animal manure. This mixture is shaped into a hexagonal solid pot (about 10 x 10 cm) and allowed to dry until it is very hard. A small depression on the upper surface of the "Morrocan pot" is seeded and filled with more soil inoculum. Several months later these container-grown seedlings which have variable quantities of ectomycorrhizae are outplanted intact. A major drawback with soil or humus inoculum is that the species of ectomycorrhizal fungi in the inoculum cannot be controlled. There is no assurance that this inoculum contains the most desirable fungi for the tree species to be produced or the site to which the seedlings are to be outplanted. Transportation of large volumes of soil inoculum is difficult. Soil inoculum may also contain harmful microorganisms and noxious weeds in addition to the ectomycorrhizal fungi. Some of these microorganisms may be potentially harmful not only to the tree seedling crop (51), but also to nearby agricultural crops (28).

Tree seedlings with ectomycorrhizae or excised ectomycorrhizae have also been used as an inoculum source for new seedling crops. In France, a truffle-producing fungus, Tuber melanosporum, has been established in nursery beds of young seedlings by transplanting seedlings with Tuber ectomycorrhizae formed under laboratory conditions into the nursery bed of new seedlings. In Indonesia, seedlings of Pinus merkusii with abundant ectomycorrhizae are planted at 1- to 2-m intervals in new seedbeds. The extramatrical mycelium developing from the "nurse" seedlings infect roots on the younger seedlings (51). Other workers (7,23) have harvested ectomycorrhizae from established trees and used them as inoculum. This successful technique has been used only on a limited basis in research trials. A great deal of time and care is required to obtain a sufficient quantity of viable ectomycorrhizae. At least 1 kg of ectomycorrhizae should be mixed with 1 m^3 of soil.

In most instances, use of the above inocula assures that most seedlings will have some ectomycorrhizae. Frequently, however, the symbiotic fungi in these inocula are not identified and their degree of benefit to the seedlings is unknown. The use of natural inoculum, however, does satisfy the first premise mentioned earlier--that any ectomycorrhizae on seedlings used in forest regeneration are better than none.

Spore Inoculum

Sporophores and spores of various fungi have been used as inoculum to form specific ectomycorrhizae on tree seedlings. Whole or chopped sporophores are dried before use. They are essentially spore inoculum, since the vegetative matrix of the sporophore is killed by dessication during drying or by decomposition when added to soil. According to Trappe (83), the first attempts to use specific fungi to form ectomycorrhizae on seedlings date back to the 18th century. Sporophores of truffle fungi were added to planting holes of oak seedlings in new plantations in attempts to enhance truffle production. These inoculations were

done nearly 75 years before the term "mycorrhiza" was coined and over 100 years before the true nature of ectomycorrhizal associations was demonstrated. There is no way of determining if these inoculations were successful.

Gastromycetes, such as the puffball-producing genera Rhizopogon, Scleroderma, and Pisolithus, produce numerous basidiospores that are easier to collect in large quantities than those of mushroom-produced ectomycorrhizal fungi. Various authors (5,20,21,60) have demonstrated the value of basidiospores as inoculum. By a variety of techniques, many scientists have successfully used basidiospore inoculum of Pisolithus tinctorius to form specific ectomycorrhizae on pine seedlings (6,29, 37,40,43,44,63). Spores of P. tinctorius are collected by crushing sporophores with ruptured peridia over a 25 to 30 mesh screen which allows the mature dry spores to pass through. Screened basidiospores are air-dried for several days at low humidity, then stored at 5°C. Spores collected and stored for several years in this fashion have been used to form Pisolithus ectomycorrhizae on pine. Since basidiospores of P. tinctorius and many other fungi will not germinate in the laboratory, spore viability can only be determined with ectomycorrhizal synthesis tests. A simple, effective inoculation procedure involves dusting dry spores of P. tinctorius onto soil around young seedlings and leaching them into the root zone with irrigation water. This inoculation has been successful on bare-root (43) and container-grown (63) pine seedlings. In most tests, 1 to 2 mg of spores per seedling have been applied. There are about 1.1×10^6 basidiospores of P. tinctorius per mg.

Inoculum composed of spores mixed with a moistened carrier, such as vermiculite, kaolin or sand, can be broadcast onto soil then mixed into the nursery soil (40,44) or mixed directly into the growing medium of containers (29,35,65) to form P. tinctorius ectomycorrhizae on pine seedlings.

Hydromulch is used to cover seeds in many tree nurseries in the United States. Hydromulch is a wood pulp or finely shredded and mildly pulped, recycled paper that is mixed with water in a special machine and blown onto seedbeds to a depth of 0.3 to 0.6 cm. Basidiospores of P. tinctorius suspended in water with a wetting agent have been mixed with hydromulch in the machine and used to cover seeds of P. taeda. Irrigation water and rain washed the spores from the hydromulch into the root zone and Pisolithus ectomycorrhizae were formed on about 75 percent of the seedlings after one growing season (40).

Several nurserymen in the United States have used another spore inoculation method. They collect sporophores of P. tinctorius in the fall and store them at 5°C. In the spring the sporophores with numerous dry spores are broken up into particles 2 to 4 mm in diameter, mixed at undetermined ratios with pine straw or sawdust, and placed as a mulch on the seedbeds. Unfortunately, this method yields highly variable quantities of Pisolithus ectomycorrhizae on seedlings at the end of the season.

Seed inoculation with basidiospores is another technique employed with some success. Theodorou (76) collected sporophores of Rhizopogon luteolus then air-dried and crushed them into a fine powder. Seeds of P. radiata were mixed with the finely divided sporophores in water. The coated seeds contained approximately 1.9×10^6 spores each. Seedlings which developed from spore-coated seeds formed abundant Rhizopogon ectomycorrhizae in sterile soil and in nonsterile soil that was deficient in ectomycorrhizal fungi. Theodorou and Bowen (78) found that inoculating seeds with spores from freeze-dried sporophores would work, but it required up to 100 times more spores to form the same quantity of ectomycorrhizae. It took 10 times more spores if the spores were air-dried for only 2 days. Freeze drying or brief air drying obviously killed or inhibited germination of many spores of R. luteolus.

Basidiospores of P. tinctorius have also been mixed with the pelletizing matrix (clay and adhesive) of encapsulated pine seeds and used to form Pisolithus ectomycorrhizae on container-grown and bare-root seedlings (Marx, unpublished data). In early tests, clay and adhesive with spores were applied too heavily (increased seed diameter by 1.5 mm) and inhibited seed germination by nearly 40 percent. When the quantity of clay and adhesive was decreased (seed diameters increased by only 1 mm), inhibition of seed germination was lowered to 20 percent. Seed encapsulation without

spores did not significantly reduce seed germination. There was no spore rate: degree of ectomycorrhizae relationship with encapsulated pine seeds. One mg of spores/seed was effective in forming Pisolithus ectomycorrhizae as were 2 to 8 mg of spores/seed. All basidiospore collections of P. tinctorius obtained from mature basidiocarps with ruptured peridia contain yeast, bacteria, and an array of other fungi. These microorganisms may cause damage to seeds and seedlings. To decrease this possibility various fungicides were added to encapsulated seeds. Two grams of captan or benomyl (50% WP) or 5.45 grams of thiram (42% EC in latex) were added per 2,000 encapsulated pine seeds. In container tests with P. taeda, captan and thiram increased and benomyl decreased Pisolithus ectomycorrhizal development on 16-week-old seedlings. Thiram and benomyl also inhibited seed germination. In a companion bare-root seedling test, neither fungicide affected seed germination or Pisolithus ectomycorrhizal development on 9-month-old seedlings of P. taeda. Earlier, captan was shown to stimulate Pisolithus and Thelephora ectomycorrhizal development on pine in fumigated nursery soils (38,45). Pisolithus ectomycorrhizae also were formed from spore encapsulated seeds of P. virginiana, P. elliottii var. elliottii, P. oocarpa, P. echinata, and P. caribaea. Tests of spore encapsulated seeds in conventional nurseries, direct-seeding trials, and inclusion of other tree species are warranted.

There are both advantages and disadvantages to using spores of ectomycorrhizal fungi for inoculation. The major advantage is that spores require no extended growth phase under aseptic conditions like vegetative inoculum (see later section). Another advantage is that spore inoculum is very light. One gram of basidiospores of Rhizopogon luteolus or Pisolithus tinctorius contains over 1 billion potentially infective propagules (basidiospores). As mentioned earlier, large numbers of basidiospores can be collected from sporophores of Gastromycetes. Over 450 kg (1,000 pounds) of mature, dry basidiospores of P. tinctorius were collected from under pine on coal spoils near Birmingham, Alabama, by personnel of the International Forest Seed Company in approximately 75 man-days. This one collection represents sufficient basidiospores to produce over 225 million pine seedlings with Pisolithus ectomycorrhizae if we assume that 1 mg of spores/seed or seedling is used. This quantity of seedlings is about one-fifth of the number grown in nurseries in the United States each year. It would be nearly impossible to collect this quantity of spores from any other ectomycorrhizal fungus, especially from fungi belonging to the Agaricales or Aphyllophorales. Another advantage of spores, at least those of certain fungi, is their ability to survive storage from one season to the next. Storability is important since spores collected in the summer or early autumn must be stored until the following spring if they are to be used to inoculate spring-sown nursery beds. The precise storage conditions for large quantities of basidiospores have not been worked out. Frequently, if the spores of P. tinctorius have a moisture content exceeding 13 to 15 percent (determined by drying at 85°C for 48 hr), growth of mold fungi will occur in cold storage (5°C) after a few months. Moldy spore collections are not very effective as inoculum. Shortly after P. tinctorius spores are collected they should be placed 1 to 2 cm deep in trays with large surface areas and air-dried at 22 to 26°C and 40 to 50 percent relative humidity. Weight loss of as much as 10 to 30 percent can occur within 60 hrs if the spores are initially moist. During drying, the spore layer in the tray should be carefully mixed every 3 to 4 hrs. Individuals processing dry spores should wear a filter mask or respirator to avoid inhaling the spores.

One of the major disadvantages of spore inoculum obtained from many ectomycorrhizal fungi is the lack of standard laboratory tests to determine spore viability. Several workers (14, 22, Marx, unpublished data) have tried a variety of physical, chemical, and biological stimuli in attempts to germinate basidiospores of P. tinctorius without reproducible results. At this time, the only reliable means of determining spore viability is through ectomycorrhizal synthesis tests. Another disadvantage is that sufficient sporophores of many fungi required to inoculate nurseries may not be available every year. For example, 450 kg of

spores were obtained from numerous sporophores of P. tinctorius produced from August through December 1980 on coal spoils around Birmingham, Alabama. From these same spoils, only 100 kg of spores were obtained during these months in 1979. The area experienced a severe drought in 1979 and in 1980 there was adequate rainfall. One would need ideal storage conditions to maintain a large inventory of spores in order to insure a constant supply of spore inoculum from year to year.

Formation of ectomycorrhizae by basidiospores usually takes 3 to 4 weeks longer than vegetative inoculum of the same fungus (40,77). This can be a disadvantage because, during this period, pathogenic fungi (26) or other ectomycorrhizal fungi often colonize the roots and reduce the effectiveness of the introduced spore inoculum. Also, seedlings experiencing a delay in ectomycorrhizal formation lose whatever growth stimulation ectomycorrhizae may give them during this period. It should be pointed out, however, that in parts of the world where the natural occurrence of ectomycorrhizal fungi is erratic or deficient, this delay may not have a significant effect on the final amount of mycorrhizae developed on tree seedlings from spore inoculations.

Perhaps the most significant problem in using spore inoculum is the lack of genetic definition. Although basidiospores of P. tinctorius collected from different sporophores in northeast Georgia did not vary significantly in their capacity to form ectomycorrhizae on pine (29), they may have varied in other traits. Genetic variation would be greater if basidiospores from sporophores collected from many geographic areas and from different tree hosts were combined into a single inoculum. Marx (32,34) demonstrated that vegetative isolates of P. tinctorius from different world locations and tree hosts varied significantly in their abilities to form ectomycorrhizae on pine and oak and to grow in pure culture at different temperatures. These same traits and undoubtedly others could vary in combined basidiospore collections. With vegetative cultures, on the other hand, certain traits can be controlled and propagated.

Vegetative Inoculum

Pure mycelial or vegetative inoculum of ectomycorrhizal fungi has been repeatedly recommended (1,33,51,69,83) as the most biologically sound method of inoculation. Unfortunately, ectomycorrhizal fungi as a group are difficult to grow in the laboratory. Many species have never been isolated and grown in pure culture. Some species grow slowly, others often die after a few months in culture. Most of these fungi require specific growth substances, such as thiamine and biotin, in addition to simple carbohydrates; most are sensitive to growth inhibitory substances (58).

The first and most important step in any inoculation program of tree seedlings is the selection of the fungi. The physiological differences are great among ectomycorrhizal fungi. These differences can be used as criteria for their selection. Host specificity is one physiological trait important to consider in the selection process. The consistent association of certain fungi with only a few specific tree hosts is well documented in the literature. Many other fungi are associated with a great number of different tree hosts (30,71,79). Any candidate fungus should exhibit the physiological capacity to form ectomycorrhizae on the desired hosts; the more hosts the better. Isolate variability within any candidate symbiont is another criterion to consider. Several isolates from different tree hosts and geographic regions should be used, at least initially, to determine the amount of variation that exists between isolates. This point has been stressed by Moser (56) and demonstrated with isolates of Rhizopogon luteolus (77), Pisolithus (32,34,52), and Paxillus involutus (18). For example, isolates of P. tinctorius from various pines were reported to form abundant ectomycorrhizae on P. taeda and Quercus rubra, but isolates from various oak species formed few ectomycorrhizae on pine or oak. Some oak isolates formed no ectomycorrhizae on either host (32,34) as did other pine isolates from Australia and Brazil (34). Another criterion is the ability of the selected fungus to grow in pure culture and

withstand manipulation. A variety of culture media (55,71,79) and methods of isolation (58) can be used to obtain pure cultures of the selected fungus. Ideally, the fungus should be able to grow rapidly. A good rate of growth in petri dish culture is a mycelial colony 6 to 8 cm in diameter after 15 to 20 days. Procedures for isolation, type of culture media, and conditions necessary for isolate storage are described elsewhere in this Manual. Once the growth characteristics of a fungus have been confirmed, it is important to determine its capacity to withstand physical, chemical, and biological manipulations. Producing large quantities of vegetative inoculum of a fungus is of little value if the inoculum cannot survive the rigors of various manipulations, such as physical processing (blending, leaching, or drying) and soil incorporation. The inoculum must also be able to survive a minimum of 4 to 6 weeks between soil inoculation and the production of short roots by the seedlings. During this period, it must survive fluctuations of soil moisture, temperature, and microbial competition.

Another criterion is the adaptation of the selected fungus to the major type of site on which the seedlings are to be planted. Of equal importance is the ability of the fungus to survive and grow under cultural conditions used in nurseries. According to Trappe (83), the ecological adaptability of an ectomycorrhizal fungus hinges on the metabolic pathways it has evolved to contend with environmental variation. Extremes of soil and climatic factors, antagonism from other soil organisms including other ectomycorrhizal fungi, pesticide application, physical disruption of mycelium from nursery operation (undercutting and root pruning), and the abrupt physiological adjustment from a well fertilized and irrigated nursery soil to an uncultivated, low fertility planting site with all its stresses are only a few of the environmental variations to which the selected fungus must adapt. The effect of temperature on different species and ecotypes of ectomycorrhizal fungi is perhaps the most widely researched environmental factor. Upper and lower temperature limits of the candidate fungi should be determined. Moser (56) studied the ability of fungi to survive long periods (up to 4 months) of freezing at -12°C and to grow at 0 to 5°C. He found that high elevation ecotypes of Suillus variegatus survived freezing for 2 months, but valley ecotypes were killed after freezing for only 5 days. The ability of certain fungi to survive freezing was not correlated with their ability to grow at low temperature, however. Generally, he did find that mountain ecotypes and species had much lower temperature optima than lowland ones. Pisolithus tinctorius can grow at 40 to 42°C (16,53) and has a hyphal thermal death point of 45°C (19). It not only survives and grows well at high temperatures but grows at 7°C (36, 42) and can overwinter in frozen soil (37). Reaction of candidate fungi to soil moisture, organic matter, and pH are also important traits to consider. Cenococcum geophilum is not only drought tolerant but forms ectomycorrhizae in natural soils ranging in pH from 3.4 to 7.5 (83). Unfortunately, the drought tolerant characteristics of C. geophilum also make it difficult to establish on pine seedlings in irrigated nurseries where it can be supplanted by Thelephora terrestris (44). Pisolithus ectomycorrhizae on pine and oak have been observed in drought-prone coal spoils ranging in pH from 2.6 to as high as 8.4. Suillus bovinus (24) and Paxillus involutus (18) form abundant ectomycorrhizae on seedlings in nurseries with high organic matter, but these ectomycorrhizae are supplanted by others after seedlings are outplanted on sites having low organic matter.

The production of hyphal strands and sclerotia are also important traits in candidate fungi. Uptake of nutrients, especially phosphorus (2), and translocation of carbon compounds (61) take place through hyphal strands. In Australia, one of the initial criteria for selection of fungi for inoculation programs is their ability to produce hyphal strands. Although research data is lacking, abundant hyphal strand production by P. tinctorius apparently enhances nutrient absorption and increases its survival potential under adverse conditions. Yellow-gold hyphal strands of this fungus, easily visible to the naked eye, have been traced through coal spoils as far as 4 meters from seedlings to sporophores by Schramm (68) and others (31). The production of sclerotia by P. tinctorius (4,46) and C. geophilum

(81) in soil or container rooting media should enhance the abilities of these fungi to survive under harsh soil conditions and, therefore, are also favorable traits in candidate fungi.

All the criteria mentioned are meaningless unless the candidate fungus is aggressive and can form abundant ectomycorrhizae on seedlings as soon as short roots are produced. It should be able to maintain superiority on seedling roots over naturally occurring fungi in the nursery. In order for tree seedlings to obtain any measurable benefit from a specific ectomycorrhizal association, there is a threshold amount of the specific ectomycorrhizal association that must be present on seedling roots. Southern pine seedlings derive little growth benefits from Pisolithus ectomycorrhizae unless half or more of all their ectomycorrhizae are Pisolithus ectomycorrhizae at outplanting on routine reforestation sites (17, 41,66). In these reports, increased growth was determined by comparing the seedlings with Pisolithus ectomycorrhizae to control seedlings with comparable amounts of T. terrestris ectomycorrhizae.

All of these criteria for selecting a candidate fungus are important, however, one must be reminded that unknown numbers of ectomycorrhizal fungi have been introduced, usually in soil inoculum, into various parts of the world to establish exotic, man-made forests(33,51). Although many species probably died, numerous fungi are currently thriving in areas halfway around the world from their original habitat. Apparently, either individually or as a group, these fungi have a tremendous capacity to adapt to different environments. Once pure culture inoculation techniques have been perfected, the value of a specific fungus should be tested over a wide range of environmental conditions. Even though the effect of a fungus on seedlings may only be temporary until it is supplanted by other fungi, the brief advantages may make the difference between survival or death of newly planted seedlings.

Several researchers in various parts of the world have developed cultural procedures for producing vegetative inoculum of a variety of fungi for research purposes. Unfortunately, large-scale nursery applications of pure mycelial cultures, even involving only a few million tree seedlings, have been severely hampered by the lack of sufficient quantities of inoculum. It is relatively simple to produce a sufficient volume of inoculum, i.e., 30 to 40 liters, for research studies carried out in small containers, pots, microplots, or even small nursery plots, but it is difficult to produce a sufficient quantity of vegetative inoculum for large-scale nursery inoculation in a practical program. A considerable quantity of vegetative inoculum would be needed to inoculate even a few southern nurseries, which together produce nearly 1 billion pine seedlings annually on some 1,400 acres of nursery soil.

Moser (54,55,56) in Austria was the first to make a serious attempt at producing vegetative inoculum of ectomycorrhizal fungi. His pioneering research has furnished the necessary basic information used by others to modify and expand upon. Moser's purpose was to develop techniques for inoculating seedlings of Pinus cembra with low temperature strains of Suillus plorans in the nursery. These strains were absent from the warmer nursery soils in the valley and in alpine meadows, but they proved to be highly beneficial to pine seedlings for reforestaation of the colder high elevation sites near the timberline.

For production of inoculum, mycelium of S. plorans was first grown in Moser's (54) nutrient solution in small flasks for several days. These mycelial cultures were then decanted into 10 liter tanks containing the same nutrient solution and aerated for 2 to 3 hours daily for 3 to 4 months. The mycelium and liquid were transferred to 5 liter flasks containing sterilized peat moss and fresh nutrient solution. During the next 2 to 4 months at laboratory temperatures, mycelium of S. plorans permeated the peat moss substrate. The inoculum was removed from the culture tanks, packaged in sterile plastic bags, transported to the nursery, and used within 3 days. Although attempts were made to maintain these cultures in aseptic condition during the 5 to 8 months incubation, contamination by Penicillium, Mucor, and bacteria often occurred. Moser (57) refers to this inoculum as

"half-pure" cultures and states that on certain occasions it proved more effective in forming ectomycorrhizae than pure cultures. He speculated that these contaminants produced a rhizosphere effect beneficial to ectomycorrhizal development and seedling growth. He tested various organic materials as the final inoculum substrate and found that forest litter or sawdust were not as effective as peat moss. He also found that agar-mycelial inoculum or mycelial suspension were not effective inocula in the nursery. Other workers (7,23,51, Marx, unpublished data) have used agar-mycelial inoculum or mycelial suspension with varying degrees of success. Moser (57) used the above technique to produce vegetative inoculum of Suillus placides, S. grevillei, S. aeruginascens, Paxillus involutus, Phlegmacium glaucopus, Amanita muscaria, and Lactarius porninsis. More recently in Austria, Göbl (9) modified Moser's technique and used mycelium to inoculate 1 liter bottles of cooked and sterilized cereal grains such as wheat or white millet. Calcium sulfate (0.4 to 0.5 g/100 g of grain) was added to improve the growth of certain fungi. These cultures were shaken lightly each week, and after 4 weeks at 20 to 22°C the grains were usually thoroughly colonized by the fungi. These grain cultures were successfully stored for up to 9 months at 4 to 6°C. During storage, they were periodically checked for microbial purity. The final inoculum was produced by adding the grain cultures to sterile peat moss enriched with nitrogen and carbohydrates (ammonium tartrate, asparagine, soybean meal, blood meal, malt extract, glucose), as well as inorganic nutrients. The kinds and amounts of these enrichments varied according to the species of fungus grown. Usually 7 to 10 grams of glucose per liter of peat moss was a standard. Ten to 15 liters of sterile, enriched peat moss were placed in large, transparent plastic bags and inoculated with 1 to 3 bottles of grain cultures. The plastic bags were plugged with cotton to provide aeration and were shaken occasionally during storage at 20 to 22°C. After 3 to 6 weeks incubation the inoculum was ready. Contaminated cultures were apparently discarded. Göbl has used this method to produce inoculum of Suillus plorans, S. grevillei, Boletinus cavipes, Amanita muscaria, and Hebeloma crustuliniforme.

Takacs in Argentina modified Moser's technique to produce inoculum for new pine nurseries established in formerly treeless areas lacking ectomycorrhizal fungi. Takacs (72,73,74) obtained isolates from sporophores and then grew them in liquid culture for several days. The mycelium was used to inoculate sterilized, germinated grains of cereals (such as barley), the cereal chaff, a mixture of grain and chaff, or peat moss. Peat moss was more commonly used than other substrates. All substrates were enriched with a liquid medium. The inoculum, regardless of substrate, was used after 1 to 2 months incubation at room temperature. Inoculum of Amanita verna, Suillus granulatus, S. luteus, Hebeloma crustuliniforme, a Russula sp., Scleroderma verrucosum, and S. vulgare have been produced using this technique. Details for a large-scale nursery inoculation in Argentina were described by Mikola (50). Usually five 200-ml flasks of each of four different fungi contained in either peat moss or grain-chaff inoculum were each mixed with 4 to 10 kg of sterilized (heat or fumigated) soil or forest litter at the nursery. These mixtures were kept moist and incubated for 3 weeks in small piles. Using this method, twenty 200 ml "starter" cultures were used to produce 100 to 200 kg of inoculum, an amount sufficient to inoculate 500 m^2 of nursery soil. Since quantitative data are not available for this work it is difficult to evaluate the success of this inoculation method. However, based on our current knowledge, it is difficult to believe that these fungi grew saprophytically in the initially sterile (probably only partially sterile) soil or litter from the cereal grain food base. This mycelium had to grow in the presence of competitive microorganisms and in the absence of essential carbohydrates. If the starter inoculum survived the soil inoculation at the nursery, perhaps all that was accomplished was a dilution of the "starter" cultures.

In Canada, Park (59) also grew pure mycelial cultures of Suillus granulatus and Cenococcum geophilum in cereal grains. Five grams of wheat grains, 100 mg of $CaCO_3$ and 8 ml of water were autoclaved in test tubes. These tubes (master cultures) were then inoculated with a fungus and incubated at 25°C for 2 to 3 weeks.

To produce mass inoculum, wheat grains were soaked in water, boiled for 30 min, and drained. Four to 5 grams of $CaCO_3$/100 g of grain were added. The grain in flasks was autoclaved, cooled, and inoculated with master cultures. After several weeks, growth of the fungi in the grain was apparent. Unfortunately, Park failed to report whether this inoculum would form ectomycorrhizae on tree hosts.

In Australia, vegetative inoculum of Rhizopogon luteolus was produced using techniques similar to those of Moser. Inoculation with R. luteolus was used to correct the deficiency of ectomycorrhizal fungi in some Australian soils and to produce seedlings of Pinus radiata with a root system having a greater capacity to absorb phosphorus from forest soil. Pure cultures were produced in a 10:2:1 ratio of vermiculite, chaff, and corn meal moistened with a liquid medium (75). The fungus was placed in bottles containing about 80 grams of medium and incubated for 1 month at 21°C. Later, Theodorou and Bowen (77) grew Suillus granulatus, S. luteus, Cenococcum geophilum, and R. luteolus in the above substrate for 3 weeks and found that it formed ectomycorrhizae on seedlings of P. radiata in pot culture.

In the United States, tests to artificially introduce pure mycelial cultures of ectomycorrhizal fungi into soil were begun by Hatch (12,13). He found that seedlings of Pinus strobus grown in prairie soil for 3 months in a filtered chamber were nonmycorrhizal and severely stunted. Half of the seedlings were inoculated with agar-mycelial inoculum of S. luteus, Boletinus pictus, Lactarius deliciosus, L. indigo, and C. geophilum. After 5 months, root evaluations revealed that S. luteus and L. deliciosus formed ectomycorrhizae and stimulated seedling growth. Noninoculated seedlings remained nonmycorrhizal and stunted. Hacskaylo and Vozzo (11) initiated a series of inoculation experiments in Puerto Rico with pure mycelial cultures of various fungi to correct a deficiency of these fungi on the island. Following Moser's (57) general technique, they grew C. geophilum, Corticium bicolor, Rhizopogon roseolus, and Suillus cothuranatus on agar medium and then in liquid medium. The mycelium from liquid culture was added to polypropylene cups containing a 2:1 ratio of sterile peat moss and vermiculite moistened with a glucose-ammonium tartrate nutrient solution (pH 3.8). After 16 weeks of incubation at room temperature in a Maryland laboratory the inoculum was transported to Puerto Rico. In the nursery, one-half cup of inoculum per seedling was placed against the nonmycorrhizal roots of 4-month-old Pinus caribaea seedlings growing in plastic bags. After 10 months, all fungi except C. geophilum formed ectomycorrhizae to varying degrees.

Marx and colleagues (33) tested various methods for producing mycelial inoculum of Pisolithus tinctorius, Thelephora terrestris, and Cenococcum geophilum. P. tinctorius was chosen as a candidate fungus because: 1) it was considered valuable in reclamation of coal spoils and other adverse sites with pines due to its natural occurrence and apparent ecological adaptation to adverse sites (46), 2) it grew rapidly in pure culture, 3) it had a broad tree host range (30), and 4) the ectomycorrhizae it formed were easily recognized and quantified on roots because of their mustard-yellow color. T. terrestris was chosen because: 1) it occurred naturally in many tree nurseries and was adapted to the good tilth and fertility of nursery soils, 2) it grew rapidly in pure culture, and 3) it had a broad tree host range (45,84). Unfortunately, Thelephora ectomycorrhizae were not very distinctive and were easily confused with the ectomycorrhizae formed by other fungi. C. geophilum was chosen because: 1) it tolerated drought and high temperature (47,48,67) and should improve seedling growth on hot, droughty sites, 2) it produced a distinctive jet-black, easily identifiable ectomycorrhizae, and 3) it had a broad tree host range (80). Unfortunately, isolates of C. geophilum grew very slowly in pure culture; a mycelial colony on agar medium of 2 to 3 cm in diameter after 3 weeks at 25°C was considered normal.

Attempts to produce effective inoculum of these fungi in wheat grains, using Park's and Takacs' techniques, failed (33). Grain cultures were added to sterilized soil in a 1:15 ratio and planted to seed of Pinus taeda in a special growth room (27). After 5 months, none of the inoculated and control seedlings had ectomycorrhizae. Microscopic examination and plating procedures revealed that the grain

cultures were colonized by saprophytic fungi and bacteria as early as 3 weeks after soil inoculation. A great deal of damping-off of pine seed was also observed. The high nutritive value of the wheat grains probably contributed to rapid colonization by saprophytes which, in turn, killed the ectomycorrhizal fungi. Macro- and microscopic examination of the grain inoculum before soil inoculations revealed that mycelium of the ectomycorrhizal fungi completely permeated the endosperm of the grain.

Vermiculite and peat moss moistened with a modification of Melin-Norkrans medium (MMN) was found to be an excellent substrate for the production of mycelial inoculum of these fungi. For decades, Melin-Norkrans medium has been the basic nutrient used in aseptic ectomycorrhizal synthesis tests. The modified formulation is simply an enrichment of this nutrient medium (25). The formulation of MMN is 0.05 g $CaCl_2$, 0.025 g NaCl, 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2HPO_4$, 0.15 g $MgSO_4 \cdot 7H_2O$, 1.2 ml of 1% $FeCl_3$, 100 µg Thiamine HCl, 3 g malt extract, and 10 g glucose in distilled water to equal one liter. Fifteen grams of agar/liter are added for agar formulation. After autoclaving, the pH of both the liquid and agar formulations is 5.5 to 5.7. This medium has proven to be as good or better than most other media for the growth of many ectomycorrhizal fungi. A ratio of 28:1 vermiculite and peat moss substrate moistened with a volume of MMN liquid medium equal to approximately half the volume of the dry substrate has proved to be the best substrate. An example is 1400 ml of vermiculite and 50 ml of peat moss mixed thoroughly and then moistened with 750 ml of MMN liquid (37). Horticultural grade No. 4 vermiculite should have all fine particles removed by screening it through a fine mesh screen (window screen). Peat moss should be finely divided and passed through the same mesh screen; the screened peat moss is mixed with the vermiculite. After autoclaving the substrate, the pH should be within the range of 4.5 and 5.5 (39).

The size of the culture vessel is not critical. One-liter Erlenmyer flasks can be used as well as 50-liter carboys. Autoclavable plastic containers of various sizes have also been used successfully. The only characteristic important in the culture vessel for these stationary, solid substrate cultures is that its dimensions should allow the vermiculite-peat moss to stay moist. Vermiculite-peat moss in a tall cylinder-like container will be dry at the top and excessively moist at the bottom after a few weeks. These extremes in moisture conditions in the medium inhibit rapid growth of ectomycorrhizal fungi. Various means of inoculating the substrate can be used. Mycelial discs from agar plate cultures or mildly blended mycelium from liquid culture work well. The more starter inoculum placed in the substrate the more rapid will be mycelial colonization of the substrate. Four to six evenly spaced agar-mycelial discs/liter of substrate are recommended. Length of incubation after inoculation with mycelial discs depends on the growth rate of the fungi. Typically, fast-growing fungi such as Thelephora terrestris and Pisolithus tinctorius incubated at room temperature will thoroughly colonize the substrate in 2-liter containers in 2 to 3 months. Slower-growing fungi, such as Cenococcum geophilum, take up to 8 to 10 months to colonize the same amount of substrate. Blended mycelial starter inoculum mixed thoroughly in the substrate will reduce the time of incubation by half. After incubation, if the fungus has completely colonized the substrate and is free of microbial contamination, the inoculum is ready for use. The latter feature can be easily confirmed by plating out portions of the substrate onto various agar media. Various other substrates, such as perlite, and 0.6-cm-diameter particles of peanut hulls, corn cobs, or pine bark, are not as suitable as vermiculite-peat moss. These fungi fail to grow in peanut, corn, or bark substrates because growth inhibitors are released during autoclaving. They will grow in perlite substrates, but the mycelium tends to grow around, rather than into, the particles which is undesirable.

Vermiculite-peat moss-nutrient inoculum as described above, when taken directly from the container and mixed into fumigated nursery soil, becomes rapidly colonized by saprophytic microorganisms. Heavy colonization reduces the effectiveness of this inoculum to form ectomycorrhizae on pine. Leaching the inoculum

before inoculation to remove most nonassimilated nutrients reduces this colonization and increases inoculum effectiveness (33). Leaching is simply done by wrapping 4 to 6 liters of inoculum in several layers of cheesecloth and irrigating this for 2 to 3 min under cool tapwater. Excess water is then removed by squeezing the inoculum in the cheesecloth by hand. Leaching removes over 65 percent of the original glucose and reduces the original inoculum volume by one-third. Leached inoculum has been used by many workers (33), but it has a very high bulk density (over 600 g/ℓ), a high moisture content (up to 91 percent), and a physical consistency of a sticky paste. These problems are corrected by drying the inoculum (38). Leached inoculum is placed 5 to 6 cm deep on wood framed wire screens (window screen) and dried at 20 to 26°C and 35 to 45 percent relative humidity. During drying the inoculum is mixed every 2 to 3 hrs to minimize excessive drying of the surface particles. Inoculum is dried at the IMRD in a room (22.6 m^3 volume) constructed of clear polyethylene plastic containing a small air conditioner, two dehumidifiers, and a small heater. Over 150 liters of dried inoculum can be processed in this room in 100 uninterrupted hours of drying. Final bulk densities range from 320 to 390 g/liter, and moisture contents from 20 to 65 percent. Final pH of inoculum ranges from 4.4 to 5.2 (45,46). The original volume of inoculum is reduced by nearly 60 percent after leaching and drying.

Dried inoculum of P. tinctorius, T. terrestris, and other fungi can be mixed more thoroughly in soil than the wetter, nondried formulations and can be effectively used at lower rates (33). Dried inoculum of P. tinctorius stored at 5°C for as long as 9 weeks and at room temperature for 5 weeks was still effective in forming ectomycorrhizae (33,45,46). However, we recommend that inoculum be dried and used as soon as possible.

A commercial formulation of mycelial inoculum of P. tinctorius has been recently developed by the USDA, Forest Service and Abbott Laboratories (45,46). This inoculum, which has been trademarked MycoRhiz®, is also grown in the vermiculite-peat moss nutrient medium. Liquid culture of Pisolithus tinctorius has recently been reviewed (70). The medium used for all liquid culture is Melin-Norkrans modified by Marx (25). Liquid culture is grown in shake flasks, 14-liter fermentors and large industrial fermentors (Plate 7) and is used as inoculum for the vermiculite-peat substrate. All liquid culture is grown with continuous agitation and high aeration rates (one volume change per minute) at temperatures of 28 to 32°C. The incubation time is dependent on inoculum level and ranges from 7 to 14 days.

Beds of vermiculite-peat moss-nutrient medium are sterilized by injection of high pressure steam in either deep-tank or rotary drum fermentors. This medium is inoculated with an actively growing liquid culture of Pisolithus tinctorius. The level of inoculum varies considerably but is usually in the range of 5-20 percent of the fermentor volume. The medium is mixed and incubated with or without agitation for 1 to 4 weeks, depending on the rapidity of growth and colonization of the substrate.

The colonized substrate is harvested by floatation in water and filtration; this also serves to wash the product form. The wet product is dried with an Aeromatic fluid bed dryer (type STS-60; Aeromatic Ltd. Muttenz-Basle, Switzerland) to a moisture level of 20-25 percent. MycoRhiz® normally has a bulk density of 240 to 310 g/liter and a pH of 5.1 to 5.6. It is packaged in 50 liter units and stored at 5°C. The shelf life under these conditions is 5 to 6 weeks. Using this fermentation technology, MycoRhiz® can be produced in sufficient volumes for large scale, practical inoculation of tree nurseries. Recently, a tractor-drawn machine was developed that will inoculate soil with MycoRhiz® and seed nursery beds in one operation (3).

During the research and development on MycoRhiz®, various assays were developed to characterize the inoculum in an attempt to predict the eventual effectiveness of the inoculum in forming P. tinctorius ectomycorrhizae in nursery soil or container substrate. One assay involved determination of propagules of P. tinctorius in inoculum. One cc of dried inoculum (final product form) was spread onto petri dishes

containing 10 ml of MMN agar medium fortified with 25 mg/liter of benomyl and 10 mg/liter of erythromycin phosphate. The plates, usually 10 per inoculum batch, were incubated at 30°C for 5 days, growth centers of the yellow-gold mycelium of P. tinctorius were counted, and counts were expressed as propagules/g of inoculum. In early trials with MycoRhiz®, certain batches had a wide range of vermiculite particle sizes (0.5 mm to 7 mm). Survival of the fungus in the smaller particles was questioned. Thus, another propagule assay was developed to determine viability of the fungus in the smaller particles. This assay involved screening inoculum through a No. 6 sieve, and placing 30 to 40 of the particles that collected on a No. 8 sieve on the surface of fortified MMN agar. These were incubated and counted as in the other propagule assay. Since microbial contamination of inoculum caused problems in previous work at the IMRD, an assay for microbial contamination of the inoculum was developed. One gram of inoculum was blended with 100 ml of sterile water for 3 min; this blend was serially diluted by factors of 10 and 1 ml lots of the diluted material were placed onto the surface of Difco trypticase soy agar. After plates, usually five per treatment, were incubated for 5 days at 30°C the bacterial and fungal colonies were counted. The dilution yielding less than 10 colonies of bacteria or fungi per plate was considered the contamination level. Since leaching of nonassimilated nutrients from the inoculum was shown to improve inoculation effectiveness in soil, a low concentration of glucose in the leached and dried inoculum was considered beneficial to inoculum effectiveness. Residual glucose (mg glucose/g of oven-dried inoculum) was determined, therefore, with an autoanalyzer by a modification of Hoffman's (15) technique.

Although MycoRhiz® effectiveness in nursery tests could not be predicted from the results of any single assay, a pattern did emerge. The most effective formulations were those with: 1) abundant hyphae of P. tinctorius inside the vermiculite particles (inoculum niche), 2) pH between 4.5 and 6.0 by having a 5 to 10 percent volume of peat moss as a component of substrate, 3) low levels of bacterial and fungal contaminants which apparently colonized inoculum during leaching and drying, and 4) low amounts of residual glucose (<16 mg/g inoculum).

CONCLUSIONS

There are a variety of methods available to ensure the development of ectomycorrhizae on forest tree seedlings for the establishment of man-made forests. Certain methods have more advantages than others. Pure mycelial inoculum has the greatest biological advantage. There is sufficient information to conclude that pure cultures of certain fungi, such as Suillus granulatus, Rhizopogon luteolus, Thelephora terrestris, and Pisolithus tinctorius, can be used to improve survival and growth of tree seedlings on a variety of sites (33,46). These results represent only the beginning of a universal practical program, however. When one considers the millions of hectares of potential exotic forests which could be established in Third World nations on former treeless lands, as well as the millions of hectares of former forested lands awaiting artificial regeneration throughout the world, the importance of the selection, propagation, manipulation, and management of superior strains or species of ectomycorrhizal fungi as a forest management tool is paramount. Research so far has only revealed the tip of the iceberg in regards to potential use of specific ectomycorrhizal fungi in world forestry. There still remains a tremendous reservoir of basic and practical information which must be revealed if these fungi are to be managed and, therefore, fully utilized in forestry. Hopefully, the techniques to produce ectomycorrhizal fungus inoculum described in this chapter will be used by challenged and foresighted plant scientists to further define and expand the value of ectomycorrhizae in forest regeneration.

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